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EVALUATION OF IMMUNE FUNCTION IN MICE EXPOSED TO ORDRAM®

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SUMMARY

The potential effects that the thiocarbamate herbicide Ordram® has on the immune system of mice was evaluated following 12 days of acute dosing by oral gavage. Dosages of Ordram® ranging from 20 to 320 mg/kg/day had no consistent significant effects on a variety of immune parameters investigated. The immune parameters measured were the following: body and lymphoid organ weights; splenic natural killer (NK) cell activity; lymphoproliferative responses to B and T lymphocyte mitogens and allogeneic spleen cells in a one-way mixed lymphocyte reaction; and delayed-type hypersensitivity and antibody responses to sheep red blood cells (SRBC). The effects that the immunosuppressant cyclophosphamide has on these immune parameters was also examined. The results indicate that Ordram® does not appear to affect key parameters of the immune system of mice under the conditions of exposure employed.

Key words: Thiocarbamate herbicide; S-ethyl hexahydro-1H-azepine-1-carbothioate; Molinate; Cyclophosphamide; Immune response; Mouse

INTRODUCTION

The thiocarbamates are a major group of herbicides used for a wide range of crops in North American agriculture because of their effectiveness in

Abbreviations: Con A, concanavalin A; CY, cyclophosphamide; DTH, delayed-type hypersensitivity; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; NK, natural killer cells; PHA, phytohemagglutinin; SRBC, sheep red blood cells.

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controlling annual grasses and their target selectivity [1]. Ordram® (molinate) is a pre-emergence thiocarbamate herbicide which is toxic to germinating broad leaved and grassy weeds and is primarily used for the control of *Echinochloa* spp. in rice [2]. Ordram® has a relatively short persistence in soil, is low in mammalian toxicity and is rapidly metabolized in rats. Approximately 97% of an oral dose is excreted via the urine (88%) and feces (11%) within 48 h [3]. While residues of [^{14}C]Ordram® associated with most tissues and organs in the rat were found to be substantially decreased 7 days after dosing, rather high levels of ^{14}C were found to persist in the blood and "blood rich" tissues such as liver, kidney, lungs, heart and spleen [3]. It is doubtful, however, that the ^{14}C residues present in blood 7 days after administration of the herbicide was due to the presence of intact parent compound [3].

The relative persistence of Ordram® or its metabolites in blood and spleen along with the fact that this and other carbamate herbicides have been reported to affect the immune system of experimental animals [4-7] led to our interest in examining the immunotoxic potential of Ordram® in mice. In this paper we report on the evaluation of immune function in mice exposed acutely (10-12 doses) to Ordram® via oral dosing. The parameters evaluated include the following: body and lymphoid organ weights; B and T cell lymphoproliferative responses; natural killer (NK) cell activity; and delayed-type hypersensitivity (DTH) and antibody response to sheep red blood cells (SRBC). The effects that dosing with Ordram® had on these endpoints were compared with those produced following a similar regimen of dosing with the immunosuppressant cyclophosphamide (CY).

MATERIALS AND METHODS

Animals and treatments

Female C57BL/6J mice, 10-12 weeks old (Jackson Lab., Bar Harbor, Maine) were housed in plastic cages on wood shavings and provided food (Wayne Lab Blox, Peoria, IL) and water ad libitum. Mice were held for at least 1 week following shipment prior to use in these studies.

Mice were dosed by oral gavage with technical grade Ordram® (kindly provided by J. Wise, Stauffer Chemical Co., Richmond, CA) in propylene glycol (Sigma, St. Louis, MO) on 12 consecutive days at dosages ranging from 20 to 320 mg/kg/day. Control mice were dosed with propylene glycol (0.1-0.2 cc) for 12 days. Mice receiving cyclophosphamide (Sigma) dissolved in distilled water were dosed by gavage on 10 consecutive days at dosages of 6-24 mg/kg/day. CY control mice were dosed with distilled water (0.1-0.2 cc).

Immune function assays

Two days following the last dosing mice were killed and their body, spleen and thymus weights recorded. Spleens were handled aseptically and cell suspensions were prepared for lymphoproliferative responses to mito-

gens and mixed lymphocyte reaction as measured by [³H]thymidine incorporation and for NK cell activity as measured by ⁵¹Cr release as previously described [8]. Briefly, spleen cells were suspended in PRMI-1640 medium, containing 25 mM HEPES, 2 mM L-glutamine, 50 µg/ml gentamycin (Grand Island Biological Co., Grand Island, NY), and 5% heat-inactivated human type AB serum (Irvine Scientific, Irvine, CA). The 72-h lymphoproliferative response to mitogens of spleen cells from individual control and treated mice was determined at optimal stimulating concentrations of the following: purified phytohemagglutinin (PHA, 0.2 µg/well, Burroughs Wellcome Co., Research Triangle Park, NC); concanavalin A (Con A, 0.1 µg/well, Difco Lab, Detroit, MI); and *Escherichia coli* 0.28:B12 lipopolysaccharide (LPS, 2 µg/well, Difco). The one-way mixed lymphocyte response of spleen cells was determined using mitomycin-C treated allogeneic spleen cells from CBA/J mice as stimulator cells in a 96-h assay [9]. All cultures were pulse-labeled with [³H]thymidine (New England Nuclear) 4 h prior to harvesting. Natural killer cell activity of spleen cell suspensions was determined using a 4-h in vitro ⁵¹Cr release assay with YAC-1 mouse lymphoma cells as targets [10]. The delayed-type hypersensitivity (DTH) and primary immune responses to SRBC were determined as described by Ron et al. [11]. Briefly, the DTH response to SRBC, was determined by measuring foot pad swelling in presensitized (4 days, intravenous injection of 0.01% SRBC) mice 24 h following intradermal injection in the hind foot pad with 50 µl of 20% SRBC. The number of splenic antibody-forming cells (IgM plaques) was measured 2 days after DTH measurement by using the Cunningham modification of the Jerne plaque assay [12].

Data presentation

The data are presented as the mean ± standard error. Data for all of the parameters tested were analyzed statistically using Dunnett's *t*-test [13]. A *P*-value of 0.01 was chosen as the level of significance.

RESULTS

The body and lymphoid organ weights of mice exposed to Ordram® or cyclophosphamide are shown in Table I. At Ordram® dosages of 160 and 320 mg/kg/day 4 of 10 mice per group died. At each of these dosages those animals which died prior to completion of the dosing regimen were observed to lose approximately 25% of their initial body weight (data not shown). The mice that died following dosing at 160 mg/kg/day died on days 6, 7 and 8, while those dosed at 320 mg/kg/day died on days 4, 5 and 6. Of those mice which survived these dosages, no difference was observed in body or thymus weight compared with control (propylene glycol) mice. While a reduction in thymus weight was observed for mice in the 320 mg/kg/day dosage group compared with control mice, this difference was not statistically significant. A significant increase in the spleen weight and the spleen to body weight ratio was observed in the 320 mg/kg/day group. No other

TABLE I

THE EFFECT OF ORDRAM® AND CYCLOPHOSPHAMIDE ON MOUSE LYMPHOID ORGAN AND BODY WEIGHTS

Treatment	Dose ^a (mg/kg day)	Mean ± S.E.				
		Body wt (g)	Spleen wt (mg)	Thymus wt (mg)	Spleen wt Body wt (×10 ⁻³)	Thymus wt Body wt (×10 ⁻³)
Ordram®	0	20.1 ± 0.3	67.1 ± 2.7	53.8 ± 2.4	3.3 ± 0.1	2.7 ± 0.1
	40	19.5 ± 0.5	70.7 ± 2.7	50.0 ± 3.1	3.6 ± 0.1	2.5 ± 0.1
	80	19.3 ± 0.4	70.5 ± 3.7	53.5 ± 4.8	3.7 ± 0.2	2.7 ± 0.2
	160 ^b	19.7 ± 0.3	72.3 ± 1.2	52.5 ± 2.5	3.7 ± 0.1	2.7 ± 0.1
	320 ^b	19.7 ± 0.2	102.2 ± 3.5 ^c	39.3 ± 3.6	5.2 ± 0.6 ^c	2.0 ± 0.2
	0	19.7 ± 0.3	78.2 ± 5.3	47.3 ± 2.7	4.0 ± 0.3	2.4 ± 0.1
	20	19.0 ± 0.2	67.0 ± 2.2	40.2 ± 1.8	3.5 ± 0.1	2.1 ± 0.1
	40	19.9 ± 0.4	77.1 ± 1.9	52.1 ± 2.4	3.9 ± 0.3	2.6 ± 0.1
	80	19.5 ± 0.3	63.7 ± 6.2	43.7 ± 2.3	3.3 ± 0.3	2.2 ± 0.1
	0	21.2 ± 0.4	72.1 ± 2.4	52.9 ± 1.3	3.4 ± 0.1	2.5 ± 0.1
Cyclophos- phamide	6	20.8 ± 3.8	75.2 ± 14.1	42.7 ± 8.4	3.6 ± 0.7	2.0 ± 0.4
	12	20.1 ± 0.4	62.5 ± 2.2	35.6 ± 3.0 ^c	3.1 ± 0.1	1.8 ± 0.1
	24	20.0 ± 0.3	65.4 ± 2.1	28.8 ± 2.2 ^c	3.3 ± 0.1	1.4 ± 0.1 ^c

^aGroups of 8–10 C57BL/6J mice dosed for 12 consecutive days with Ordram® or 10 consecutive days with cyclophosphamide by oral gavage.

^bFour of 10 mice dosed with Ordram® at these doses died prior to assay.

^c $P < 0.01$, Dunnett's *t*-test vs. control.

differences in body or lymphoid organ weights were seen in this experiment or in a subsequent experiment with an 80 mg/kg/day maximum dosage. In contrast, mice dosed at 12 and 24 mg/kg/day CY had significant thymic involution compared with controls (water), but no change in body or spleen weight.

Table II shows the lymphoproliferative response to mitogens and allogeneic cells of mice dosed with Ordram® or CY. Extremely low [³H]thymidine incorporation was observed for Con A, PHA and LPS stimulated splenocytes, while MLR responses were normal, in the first experiment shown in Table II. The reason for this poor response is not known. However, in a subsequent experiment, at sublethal doses of Ordram®, normal responses to these mitogens were observed. No significant differences were observed between Ordram® exposed and control mice to the B cell mitogen LPS, the T cell mitogens PHA and Con A and the T cell response to allogeneic cells. In contrast, mice exposed to CY had suppressed responses to Con A, PHA, LPS and allogeneic cells compared with controls. However, significant

TABLE II

LYMPHOPROLIFERATIVE RESPONSES TO MITOGENS AND ALLOGENEIC CELLS,
AND NATURAL KILLER CELL ACTIVITY OF SPLEEN CELLS FROM MICE
EXPOSED TO ORDRAM® OR CYCLOPHOSPHAMIDE

Treatment	Dose ^a (mg/kg day)	Mean CPM ($\times 10^{-3}$) $\pm$ S.E.				Mean % ⁵¹ Cr released $\pm$ S.E.
		Con A	PHA	LPS	MLR	NK activity ^c
Ordram®	0	1.7 $\pm$ 0.2	3.1 $\pm$ 0.3	1.8 $\pm$ 0.2	13.8 $\pm$ 0.8	11.1 $\pm$ 0.8
	40	2.0 $\pm$ 0.4	3.1 $\pm$ 0.5	1.7 $\pm$ 0.3	20.1 $\pm$ 2.5	12.6 $\pm$ 0.9
	80	1.7 $\pm$ 0.2	2.6 $\pm$ 0.2	1.5 $\pm$ 0.2	17.5 $\pm$ 0.5	13.3 $\pm$ 3.7
	160 ^b	2.5 $\pm$ 0.3	3.1 $\pm$ 0.4	1.9 $\pm$ 0.2	19.4 $\pm$ 2.3	13.4 $\pm$ 1.8
	320 ^b	1.9 $\pm$ 0.2	1.9 $\pm$ 0.2	1.1 $\pm$ 0.2	15.8 $\pm$ 0.8	12.5 $\pm$ 1.0
	0	49.6 $\pm$ 6.6	47.8 $\pm$ 5.2	15.5 $\pm$ 1.2	13.3 $\pm$ 1.1	11.7 $\pm$ 0.7
	20	54.0 $\pm$ 4.6	56.9 $\pm$ 3.7	19.5 $\pm$ 1.3	14.2 $\pm$ 1.2	13.1 $\pm$ 0.7
	40	67.1 $\pm$ 6.5	63.9 $\pm$ 3.8	19.8 $\pm$ 1.6	14.9 $\pm$ 1.5	12.2 $\pm$ 0.9
	80	62.3 $\pm$ 4.2	58.6 $\pm$ 2.9	18.7 $\pm$ 1.6	14.3 $\pm$ 1.4	12.3 $\pm$ 0.9
	0	134.7 $\pm$ 1.7	64.0 $\pm$ 3.0	57.5 $\pm$ 6.5	30.1 $\pm$ 5.6	20.3 $\pm$ 4.2
Cyclophos- phamide	6	124.6 $\pm$ 2.7	61.0 $\pm$ 4.7	56.6 $\pm$ 2.9	18.5 $\pm$ 2.0	14.2 $\pm$ 0.9
	12	119.8 $\pm$ 3.3	66.2 $\pm$ 4.9	37.9 $\pm$ 4.2	22.7 $\pm$ 2.3	16.1 $\pm$ 1.0
	24	81.8 $\pm$ 12.2 ^d	40.5 $\pm$ 8.0 ^d	31.1 $\pm$ 4.7 ^d	19.2 $\pm$ 3.6	13.2 $\pm$ 3.7

^aGroups of 8–10 C57BL/6J mice dosed for 12 consecutive days with Ordram® or 10 consecutive days with cyclophosphamide by oral gavage.

^bFour of 10 mice dosed with Ordram® at these doses died prior to assay.

^c100:1 effector to target cell ratio.

^d $P < 0.01$, Dunnett's *t*-test vs. control.

TABLE III

DELAYED TYPE HYPERSENSITIVITY AND ANTIBODY RESPONSES TO SHEEP
RED BLOOD CELLS IN MICE EXPOSED TO ORDRAM® OR CYCLOPHOSPHAMIDE

Treatment	Dose ^a (mg/kg/day)	DTH response ^b footpad swelling (mm $\times 10^{-1}$)	Antibody response ^b	
			PFC/10 ⁶ Cells	PFC/Spleen ($\times 10^{-4}$)
Ordram®	0	17.3 $\pm$ 1.8	6926 $\pm$ 861	115.2 $\pm$ 17.5
	80	17.3 $\pm$ 1.1	6406 $\pm$ 355	93.9 $\pm$ 4.4
Cyclophosphamide	0	18.3 $\pm$ 1.3	9150 $\pm$ 964	154.8 $\pm$ 29.6
	24	5.6 $\pm$ 1.0 ^c	3893 $\pm$ 901 ^c	51.9 $\pm$ 13.1 ^c

^aGroups of 8 C57BL/6J mice dosed on 10 consecutive days.

^bResults are expressed as mean $\pm$ S.E.

^c $P < 0.01$, Student's *t*-test vs. control.

differences ($P < 0.01$) were seen only in the Con A, PHA and LPS but not MLR response of cells from mice dosed at 24 mg/kg/day. NK cell activity was unaffected by Ordram® and reduced, albeit not significantly, by CY.

The DTH and antibody responses to SRBC as measured by foot pad swelling and as IgM plaque forming cells were unaffected by exposure to 80 mg/kg/day Ordram® (Table III). Significant suppression of both the DTH and antibody responses was, however, observed in mice dosed at 24 mg/kg/day CY.

DISCUSSION

The effects that a variety of carbamate pesticides and herbicides have on the immune system of mammals has recently been documented in a review by Street [14]. Effects following exposure to carbamate compounds ranged from reductions in non-specific immunity and impairment in cellular and humoral immunity to reductions in resistance to infection with bacteria [14].

Immune function effects have been reported in the literature to occur in rats dosed over a period of 4–5 months with the carbamate herbicide molinate [4–7]. The bactericidal capacity of serum and neutrophils was reported to be suppressed in rats receiving a "toxic dose" ($1/20 \text{ LD}_{50}$) of molinate [5]. Decreased concentration of serum lysozyme were reported to occur in the progeny of rats dosed for 3–4 months with molinate [7]. Treatment of rats for 4–5 months with molinate at a dosage of $1/20 \text{ LD}_{50}$ but not $1/1000 \text{ LD}_{50}$ molinate resulted in suppression of antibody production following immunization [6].

In the present study no significant immune effects were observed in female C57BL/6J mice dosed acutely with Ordram®. At dosages of 160 and 320 mg/kg/day 40% of the mice died, apparently due to the general toxic effects of Ordram® at these dosages. At a dosage of 80 mg/kg/day, the highest tolerated dosage in this study, no differences were observed in body or lymphoid organ weights, lymphoproliferative responses of T and B lymphocytes, NK cell activity, DTH response, or antibody plaque forming response compared with control mice. Similarly, no effects were observed in male CBA/J mice exposed to dosages of Ordram® as high as 80 mg/kg/day for 12 days (data not shown). In comparison, the potent immunosuppressant cyclophosphamide [15] caused significant thymic involution and significantly reduced the lymphoproliferative responses of both T and B lymphocytes as measured in the mitogen-stimulated response. CY also inhibited the delayed type hypersensitivity responses to SRBC and the antibody response to the T lymphocyte dependent antigen SRBC. While CY (24 mg/kg/day) reduced the MLR and NK cell activity of mice by approximately 35% these reductions were not significant. The failure of CY to significantly suppress the MLR may be evidence that the T cells

which participate in the MLR may be more resistant to the cytotoxic effect of CY than are the T cells which are Con A and PHA responsive [16]. Similarly, the failure to suppress NK activity with CY may also result from the fact that NK cells appear to be less sensitive to CY than B and T lymphocytes [17].

The results of this study indicate that acute exposure of mice to Ordram®, under the conditions described, does not affect immune function. Whether chronic exposure (i.e., 4–5 months) to this herbicide might alter immune function, as reported elsewhere [4–7], remains to be corroborated.

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